

and under their supervision. Consideration of the data presented in the manuscript is requested.

Also in response to the Examiner's rejection under 35 USC 101, Applicants directed the Examiner's attention to several references that demonstrate that protection against HIV infection *in vivo* correlates with *in vitro* assays (see page 4 of the Amendment filed December 23, 1993). Copies of the cited documents are attached and the portions that are of particular relevance have been highlighted. The Examiner is requested to consider with care the reference of Girad et al. The assay described on page 543 of that reference is a syncytia forming assay comparable to the one used by Applicants.

Also enclosed are sections of a general HIV methodology text. Particular attention is directed to the section entitled "Evaluation of Candidate Anti-HIV Agents In Vitro" (page 225). The outline provided there is roughly that which Applicants followed (syncytia formation, RT assays and p24 levels). Implicit in the teachings of this book is that *in vitro* assays will be predictive of anti-HIV activity *in vivo*.

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In view of the foregoing and attached, the Examiner is again urged to consider the rejection under 35 USC 101 and withdraw same.

Respectfully submitted,

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Full title:

**Inhibition of HIV-1-BaL Infection of  
Mononuclear Phagocytes by Anti-CD44 Antibodies\***

by

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### Abstract

Cellular CD4 is the primary membrane molecule that binds HIV-1 through interaction with viral gp120. Membrane glycolipids and cell adhesion molecules have also been noted to be involved in the interaction of HIV-1 with cells and in syncytia formation in infected cells. The purpose of this study was to determine the role of the cell adhesion molecule CD44 in HIV-1 infection of cells. Both normal blood monocytes and lymphocytes expressed CD44 as determined by flow cytometry using the anti-CD44 antibody A3D8. Anti-CD44 monoclonal antibodies A3D8 and A1G3 inhibited infection of monocytes and peritoneal macrophages with HIV-1-BaL, but had no effect on HIV-1-IIIB infection of mitogen-stimulated lymphocytes, or cells of the T lymphocyte line. CD44 monoclonal antibodies were not toxic for monocytes, and the observed inhibitory effect of CD44 monoclonal antibodies was not dependent on complement. Hyaluronic acid, one ligand of CD44, also inhibited HIV-1-BaL infection of monocytes. These results suggest that CD44 may be a determinant of HIV-1 infection of mononuclear phagocytes *in vitro*.

## Introduction

Cellular CD4 has been recognized as the predominant membrane protein that interacts with HIV-1. In most cells, CD4 serves as the receptor that binds HIV-1 gp120 initiating events that lead to cell-viral membrane fusion and internalization of HIV into the susceptible cell (1-3). The ability of HIV to infect some CD4 negative cells (4-6), and the observation that gp120-CD4 interactions may not be the sole determinants of HIV-1 infectivity and spread (7) have prompted the search for additional cell surface molecules involved in HIV-1 infection. For example, although the lymphocyte function associated-type 1 (LFA-1) molecule has been shown not to be an important determinant of HIV-1 infection of cells, it is required for efficient fusion of HIV-1-infected cells and syncytia formation (8-12). Furthermore, in CD4 negative neural cell lines and colon epithelial cells, investigators have found that the membrane lipid galactosyl ceramide can serve as a receptor for HIV-1, and bind gp120 (5, 6). Also, investigators have noted that even though certain viral strains preferentially infect either monocytes or lymphocytes (monocytotropic or lymphocytotropic viral strains), both strains interact with CD4 (13, 14). This implies that other cellular molecules may play a role in determining tropism.

To determine the role of non-CD4 cell membrane molecules in HIV-1 infection of monocytes and lymphocytes, we have been studying various anti-cell adhesion molecule antibodies in *in vitro* assays of HIV-1 infection. We report here that antibodies against CD44 effectively inhibit the infection of human mononuclear phagocytes (monocytes and peritoneal macrophages) with the monocytotropic HIV-1 strain BaL. However, the antibodies have no effect on the infection of normal blood lymphocytes and a T lymphoid cell line with the lymphotropic HIV-1 strain IIIB. These studies indicate that CD44 may be an important determinant of HIV-1 infection of mononuclear phagocytes.

## Methods and materials

*Cells:* Human monocytes, peritoneal macrophages, lymphocytes, and blood mononuclear cells were isolated by sequential ficoll-Hypaque and Percoll gradient centrifugations as noted before

(15-17), under a protocol approved by the Duke University and VA Medical Centers Institutional Review Boards. Monocytes and peritoneal macrophages, after isolation, adherence, and washing were greater than 95% pure, as determined by Wright's and nonspecific esterase stains.

Mononuclear phagocytes were cultured with Dulbecco's modified Eagle medium of low endotoxin content and 10% human serum. The continuous human T lymphoid cell line CEM is maintained in our laboratory and is mycoplasma free. Normal blood lymphocytes activated by phytohemagglutinin and interleukin-2 were prepared as noted before (17). Lymphocyte cultures were done in RPMI-1640 medium of low endotoxin content with 10% heat inactivated fetal bovine serum. Cell surface antigen analysis by indirect immunofluorescence using a FACSTAR analyzer was done as described before (18).

*Viral strains:* HIV-1-BaL (monocytotropic) and HIV-1-IIIB (lymphocytropic) viral strains were used (19, 20). Cells were infected at a multiplicity of infection of approximately 0.01 to 0.1.

*Monoclonal antibodies:* Murine monoclonal antibodies directed against CD44 [A3D8 and A1G3 (21, 22)], and MHC class I (3F10) were used as ascites or as IgG purified using a Staph protein A column. Monoclonal antibodies A3D8 and A1G3 recognize different epitopes of the CD44 molecule (21, 22). Ascites from mice bearing the parent mouse myeloma cell line was used as control (P3) in some experiments. Antibodies were added to the cells at initiation of the cultures, and left in throughout the culture period. Cells were inoculated with virus within 24 hours of initiation of the experiments. Supernatant media were collected every 3 to 7 days, and terminated 18 to 21 days after inoculation.

*Assessment of viral infection:* Morphologic cytopathic effects were determined by observation of live cells with inverted phase contrast microscopy, and of methanol-fixed cells stained with Wright's stain at the end of the experiments. Reverse transcriptase was measured after 14 days of culture as noted before determining counts by scintillation counting or by the use of a phosphoimager (17, 23). Reverse transcriptase results from a typical experiment using

monocytes and HIV-1-BaL revealed a mean of 50125 cpm for monocytes with HIV-1-BaL, while those for lymphocytes using HIV-1-IIIB were 90518 cpm.

*Other methods and reagents:* All other reagents were from Sigma Chemical Company (St. Louis, MO). The presence of endotoxin was determined by the limulus amebocyte lysate colorimetric assay (Whittaker Laboratories, Bar Harbor, ME).

## Results

CD44 is expressed by numerous cell types, including erythrocytes, lymphocytes, and mononuclear phagocytes (24). As seen in figure 1, the anti-CD44 antibody A3D8 reacted strongly with normal blood lymphocytes and monocytes isolated by density gradient sedimentation as noted before (15) (figure 1). Monocytes had a slightly greater density of CD44 than did lymphocytes.

As noted before by us and others, HIV-1-BaL infection of human monocytes and peritoneal macrophages resulted in cytopathology (multinucleated giant cells), and in the production of supernatant viral RT and p24. If the cultures were done in the presence of antibodies against CD44, there was a consistent decrease in the HIV-1-induced cytopathology (figure 2) and of RT levels in culture supernatant medium (figure 3). The antibodies against CD44 inhibited HIV-1 infection of monocytes, while an antibody against MHC class I antigen (3F10) did not appreciably alter the RT levels (figure 3). The inhibition was seen when using ascites or purified A3D8 IgG (figure 3).

Investigators have noted that as mononuclear phagocytes differentiate [either *in vitro* or *in vivo* (23, 25)], they become more susceptible to productive infection with HIV-1. We found that freshly isolated peritoneal macrophages [the *in vivo* differentiated form of blood monocytes (23)], like monocytes, were inhibited in their ability to be infected with HIV-1-BaL by monoclonal antibodies against CD44 (figure 3). To determine the possible role of complement in the inhibition, we cultured monocytes in serum which had been heated to inactivate complement.

Under these culture conditions, the anti-CD44 antibodies inhibited HIV-1-BaL productive infection to a comparable degree (75 to 80% inhibition). As opposed to the inhibition of HIV-1 infection of mononuclear phagocytes with anti-CD44 antibodies, these antibodies had no inhibitory effect on HIV-1-IIIB infection of proliferating blood lymphocytes (figure 3).

Likewise, the anti-CD44 antibody A3D8 did not inhibit HIV-1-IIIB infection of cells of the T lymphocyte cell line CEM. For example, eight days after inoculation with undiluted HIV-1-IIIB, supernatants from control CEM cells had RT values of 332,758 cpm (100%), while those with A3D8 (1:100 dilution) were 373,896 (112%).

Certain isoforms of CD44 are known to bind hyaluronan (hyaluronic acid) and to serve as the cellular receptor for this extracellular matrix component (24, 26). To determine if soluble hyaluronan could inhibit infection of monocytes, we incubated varying amounts of hyaluronan with monocytes and determined the ability of HIV-1-BaL to infect these cells. Hyaluronan inhibited HIV-1-BaL infection of monocytes, with an ID<sub>50</sub> of approximately 5 ug/ml. Other extracellular matrix components had no or little inhibitory effect (figure 4). However, hyaluronan did not alter HIV-1-IIIB infection of normal peripheral blood lymphocytes or CEM cells (data not shown).

## Discussion

In this study, we have demonstrated that productive infection of human monocytes with HIV-1-BaL (as manifest by cytopathology and production of viral RT) can be inhibited by antibodies against the membrane glycoprotein CD44 and by the CD44 ligand hyaluronan. However, infection of CD44 positive, mitogen-activated T lymphocytes or CEM cells with the lymphotropic virus HIV-1-IIIB was not inhibited by the CD44 monoclonal antibodies or by hyaluronan.

Human CD44 (previously known as Pgp-1, HCAM, Hermes antigen, and the lymphocyte homing receptor) is now known to be a family of related glycoproteins of different function formed apparently by alternative splicing of RNA (24, 27-30). These different isoforms may



mediate various functions including (i) serving as the membrane receptor for hyaluronan; (ii) anchoring cells to the extracellular matrix by binding hyaluronan, fibronectin, or collagen; (iii) binding to the cytoskeletal protein ankyrin; (iv) mediating leukocyte binding to endothelial cells, and leukocyte aggregation; (v) serving as a leukocyte receptor involved in lymphocyte co-mitogenesis and monocyte monokine secretion; and (vi) in determining metastatic behavior of certain tumor cells (24). Soluble CD44 has been described in tissue fluids and plasma (31), and soluble CD44 or CD44 in liposomes can apparently interfere with the normal function of CD44 *in vitro* or *in vivo* (31-33). An intact CD44 cytoplasmic domain is critical for the functional activity of CD44 (26); interaction with protein kinase C may be important in the cell signaling pathways (34, 35).

Gallatin and co-workers noted earlier that in macaques infected with simian immunodeficiency virus (SIV), there was a selective depletion of CD4 positive-CD44 ("heterotypic adhesion receptor") "high" cells (36). Furthermore, the CD4<sup>+</sup>,CD44<sup>hi</sup> cells were much more susceptible to productive SIV infection *in vitro* (36). This suggests that CD44 might be a determinant of lentivirus infection of cells *in vivo*. Our experiments demonstrate that monocyte CD44 could be involved in the *in vitro* HIV-1 infection of human monocytes. The mechanism(s) by which anti-CD44 antibodies inhibit this infection is not known. It is possible that monocyte CD44 serves as an auxiliary binding molecule for HIV-1 membrane components (e.g., gp120). Alternately, engaging CD44 with either antibody or ligand (hyaluronan) could cause "activation" of monocytes to express other anti-viral mechanisms [e.g., expression of interferon-alpha (37, 38)]. Expression of different CD44 isoforms in various cell types (e.g., monocytes and lymphocytes) could determine cellular responses to anti-CD44 antibodies and hyaluronan, and explain the differences we note in this inhibition of HIV-1 infection of lymphoid cells and monocytes. Our findings suggest that *cellular* CD44 (in addition to cellular CD4 and viral gp120) may be an important determinant of viral tropism.

The full elucidation of the mechanism(s) underlying the observed inhibition may aid in the understanding of HIV-1 infection. This work may help in the development of novel strategies for preventing or controlling HIV-1 infection; anti-CD44 antibodies, CD44 ligands, or soluble CD44 could prove useful therapeutically.

## **Acknowledgments**

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### Figure legends

Figure 1—Flow cytometric analysis of blood lymphocytes and monocytes using with anti-CD14 and anti-CD44 monoclonal antibodies. Monocytes and lymphocytes were separated by sequential centrifugation as described in the Methods, and analyzed for expression for CD14 with antibody LeuM3 (panel A) and CD44 with antibody A3D8 (panel B). The solid line is for monocytes, and the dotted line is for lymphocytes.

Figure 2—Photomicrograph of control and HIV-1-BaL-inoculated monocytes without or with monoclonal anti-CD44 antibody A3D8. Monocytes were placed into microtiter wells  $\pm$  antibody  $\pm$  HIV-1-BaL, and cultured for 21 days. The anti-CD44 monoclonal antibody A3D8 prevents the cytopathic effect caused by HIV-1-BaL.

A, control monocytes; B, control monocytes inoculated with HIV-1-BaL; C, A3D8-treated control monocytes; D, A3D8-treated monocytes inoculated with HIV-1-BaL. (Wright's stain; original magnification 40X).

Non-inoculated monocytes (A and C) are generally mononuclear; while those inoculated with HIV-1-BaL and cultured without antibody A3D8 contain numerous multinucleated giant cells (syncytia) with 4 to 100 nuclei (B). HIV-1-BaL-inoculated monocytes cultured with antibody A3D8 have no multinucleated giant cells (syncytia) (D).

Figure 3—Inhibition of HIV-1-BaL productive infection of blood monocytes and peritoneal macrophages (but not blood lymphocytes) by anti-CD44 monoclonal antibodies. The data is expressed as percent of control for the reverse transcriptase values measured 14 days after inoculation.

- = control;
- ▲ = monocytes with unheated human serum;
- ◆ = peritoneal macrophages with unheated human serum;
- = monocytes with heat-inactivated human serum;
- × = monocytes with purified A3D8 antibody;
- ▶ = peripheral blood lymphocytes
- = mean

Figure 4—Inhibition of HIV-1-BaL productive infection of blood monocytes by hyaluronic acid. Monocytes were seeded with various doses of hyaluronic acid or chondroitin sulfate (SO<sub>4</sub>), and inoculated with HIV-1-BaL. Supernatant reverse transcriptase was measured after 14 days of cultures. This shows results of a representative experiment expressed as percent of control for the reverse transcriptase values.

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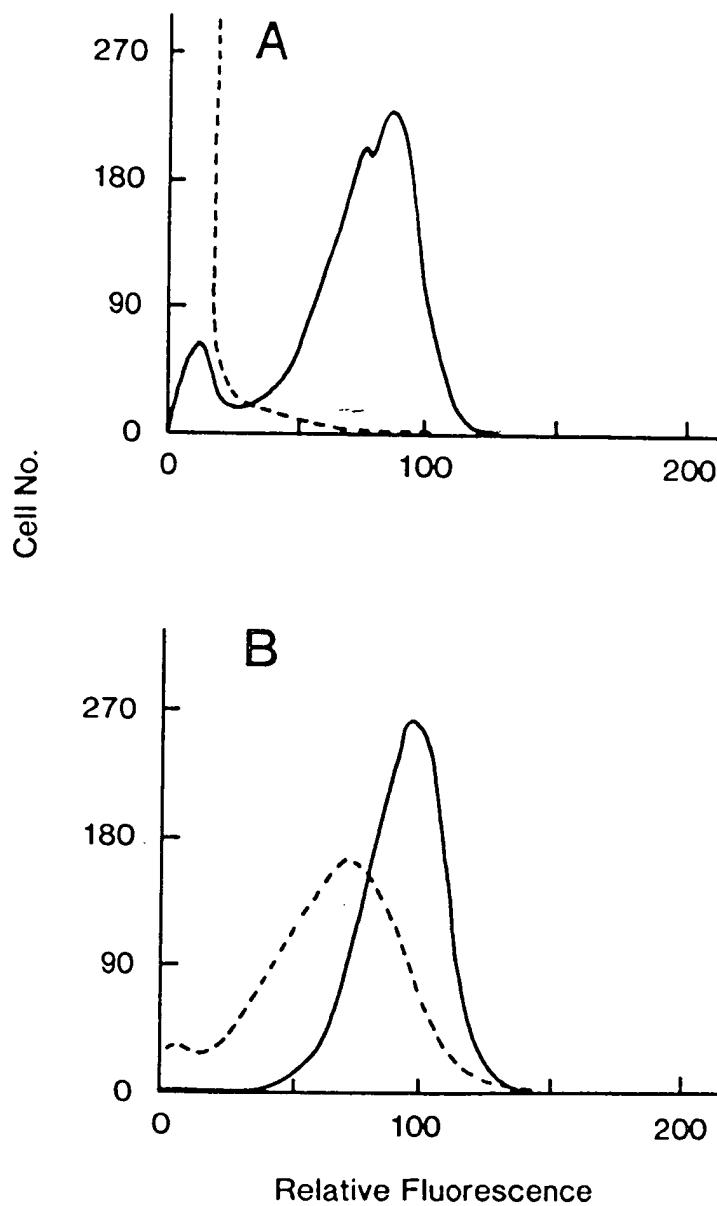


Fig 1 - *Pyrobaculum*, 1st

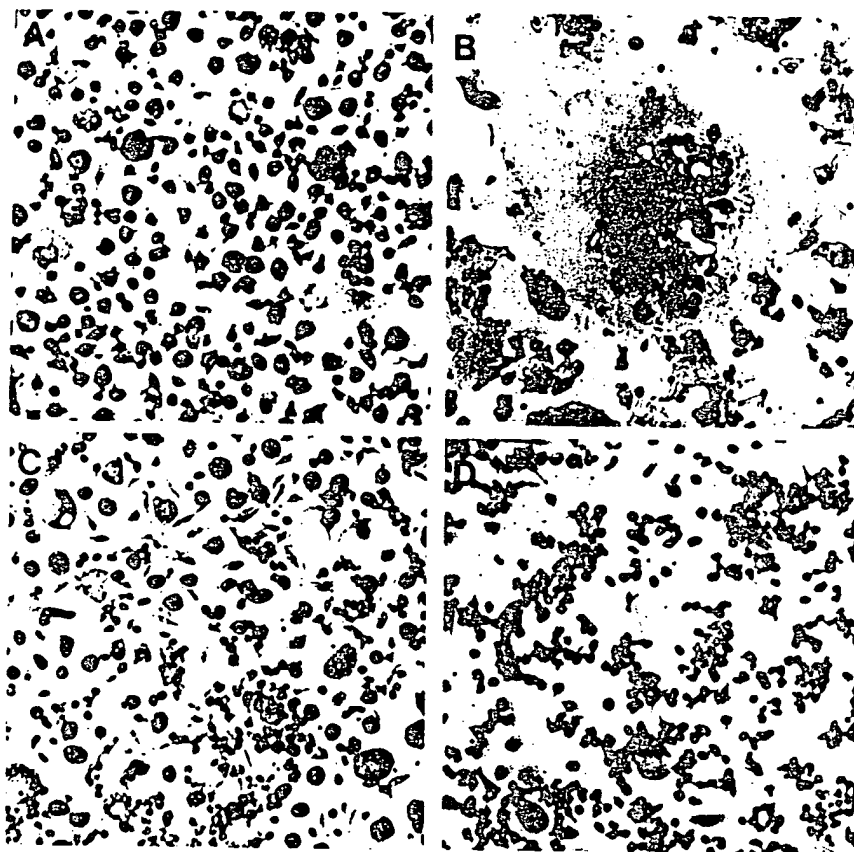


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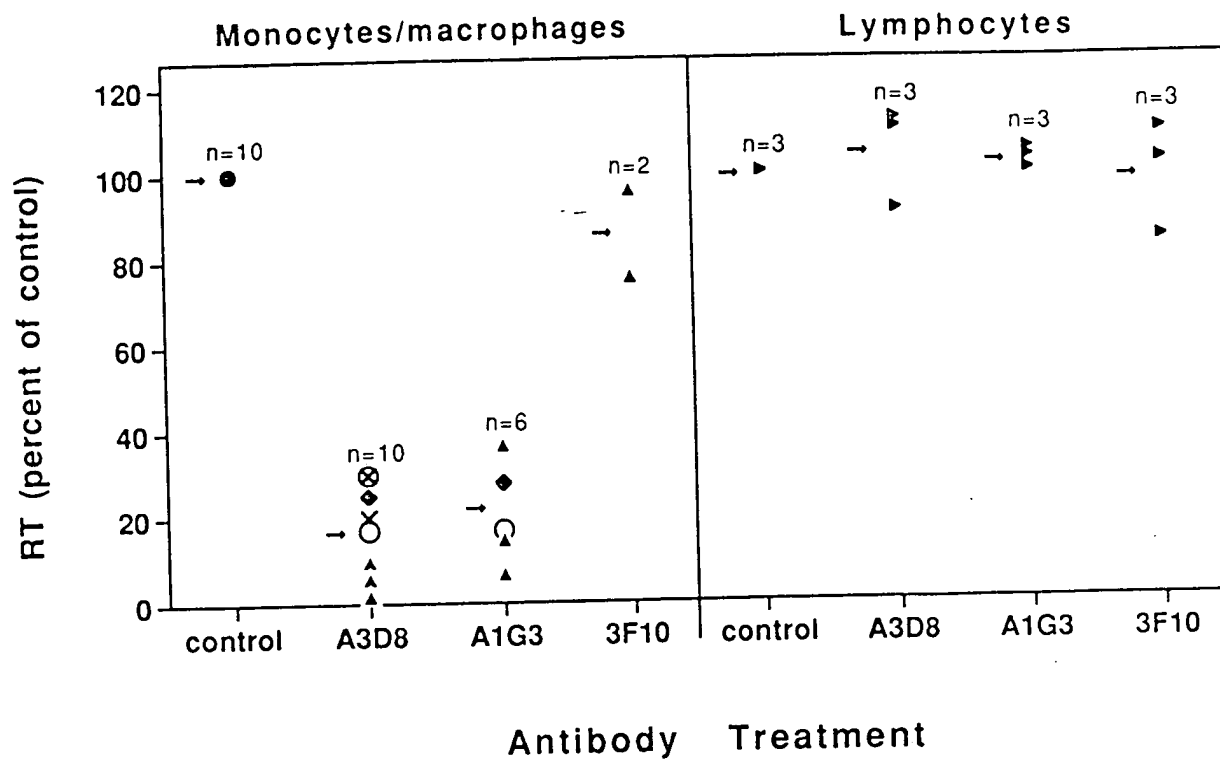


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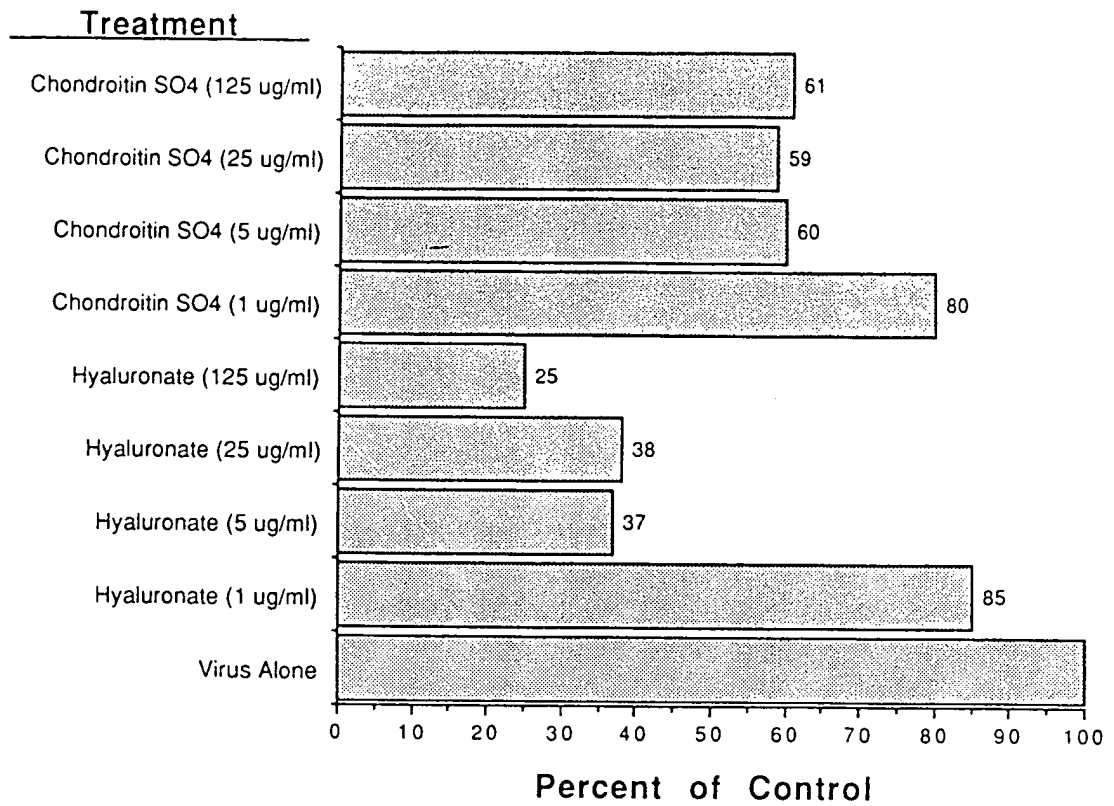


Fig 4  
Resistant virus, et al